**Original Research Article**

# Characterisation of Susceptibility of Candida Spp. to Three Essential Oils – A Study done in FIMS, Kadapa

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## Abstract

In the present investigation, anti-Candida activity of three essential oils that is Betel leaf (*Piper betel*), Black cumin (*Nigella sativa*) and Curry leaf (*Murraya koienigii*) were screened against three human pathogenic species of Candida namely *Candida albicans*, *Candida glabrata* and *Candida tropicalis*. The minimum inhibitory concentration (MIC) values of the oils ranged between 14.80 and 236 µl/ml while studied through the dilution method. The oils retained their anti-Candida activities even after heat treatment (at $45^0$C, $60^0$C, $100^0$C for 1 hour) and also on autoclaving. Black cumin leaf oil showed better anti-Candida activity against *Candida albicans*, resulting in an irreversible damage to the cells. The anti-Candida activity of these essential oils could be attributable to the membrane inhibition mechanism. The activity of the cells is reported to be microbicidal.

## Key words

Anti-Candidal activity, Essential oils, Minimum inhibitory concentration.

## Introduction

For the past ten years fungal infections are increasing and a rise in resistance to fungicides for most of the species are seen in routine medical practice. Of all the hospital acquired blood stream infections Candida species

infections are fourth leading cause. Leading to mortality of up to 40% for systemic and disseminated infections. Clinical manifestations include oropharyngeal infections and infections among persons with Human Immunodefficiency Viruses (HIV) or full blown disease of Acquired Immunodefficiency Syndrome (AIDS) patients and also candidaemia, vulovaginal infections affecting women of all age groups [1-5].

About 50% of both superficial and systemic mycoses and almost all the mucosal candidiasis is caused by *Candida albicans*. However serious oropharyngeal candidiasis and occasional esophageal candidiasis were caused by non albican Candida species such as *Candida glabrata, Candida tropicalis, Candida parapsilosis* and *Candida krusei.*

There is increased interest generated among the academicians and researchers towards herbal medications because of development of drug resistance towards the commonly used antibiotics and chemotherapeutic agents. For centuries, the therapeutic properties of various medicinal plants have been used to treat human diseases. It has been estimated that between 50% to 80% of the populations of developing countries use traditional and botanical medicines almost exclusively and consider them to be a normal part of primary healthcare [6-11]. Essential oils derived from plants are well known in traditional medicine and proved to have insecticidal, bactericidal, fungicidal and nematicidal effects. Antibacterial activity of essential oils are well cited in literature. However, the antifungal activity of essential oils specifically against Candida species is less studied. Because of that this study has been done. So, a study in Fathima Institute of Medical Sciences, Kadapa was done in September 2013.

## Materials and methods

- Betel leaf (*Piper betel*)
- Black cumin (*Nigella sativa*)
- Curry leaf (*Murraya koienigii*)
- Hydro-steam distilled essential oils derived from Southern Spice Pvt. Ltd. Madurai and used for this study.
- Fungal Pathogens: -
- Human pathogenic species of Candida such as –
- *Candida albicans*
- *Candida glabrata* and
- *Candida tropicalis*

Above species were maintained on Sabouraud's Dextrose Agar slants in the laboratory.

**Minimum Inhibitory Concentration (MIC) – Determination**

Minimum Inhibitory Concentration (MIC) of the oils were determined by double dilution method [14] in Sabouraud's Dextrose Broth supplemented with Tween 20 (0.75%) to facilitate miscibility of oils.

**Minimum Killing time of Oils – Determination**

To determine the Minimum Killing Time of the oils against the test pathogens, 1 ml of Sabouraud's Dextrose Broth with Tween – 20 (0.75%), at the MIC level of the oils was prepared and inoculated with 0.1 ml of freshly grown test organisms and incubated at $5^0$C, room temperature ($25\pm2^0$C) and $37^0$C respectively. One loopful of the sample from the above test tubes were subcultured onto SDA plates at 0, 5, 10, 15, 30, 45, 60, 120, 180, 240, 300, 360, 420, 480 minutes intervals and incubated overnight. Two sets of tubes were incubated for each test organism at a specific temperature from which subculturing was carried out alternatively to minimize the time lapse during subculture. The activity was observed after overnight incubation of the plates at $25^0C\pm2^0$C. Absence of growth on the streak line was considered to be the time taken by the oil to kill the organism.

**Effect of pressure and temperature on anti-Candida activity of the oils – Determination**

The effect of pressure and temperature was studied by autoclaving at $121^0$C for 20 minutes and heating the oils at different temperatures that

is $45^0$C, $60^0$C and the activity was studied at MIC levels of the oils.

## Results

**Minimum Inhibitory Concentration**

Minimum Inhibitory Concentration (MIC) of oils ranged from 14.80 to 236 µl / ml (**Table – 1**). Lowest MIC value of 14.8 µl/ml was reported in case of *Nigella sativa* oil against *Candida albicans* and *Candida tropicalis*. *Murraya koenigii* showed MIC value 128 µl / ml against *Candida albicans* and *Candida glabrata* whereas, the same oil showed higher MIC values of 236 µl / ml against *Candida tropicalis*.

**Table – 1:** Minimum Inhibitory Concentration of the oils against test pathogens.

| **Pathogens** | **MIC (µl / ml) of oils** | | |
|---|---|---|---|
| | ***Piper betel*** | ***Nigella sativa*** | ***Murraya koenigii*** |
| *Candida albicans* | 127.0 | 14.80 | 128.0 |
| *Candida glabrata* | 58.25 | 42.15 | 126.2 |
| *Candida tropicalis* | 28.70 | 14.80 | 236.0 |

MIC: Minimum Inhibitory Concentration

**Fungicidal / Fungistatic nature of oils – Determination**

By subculturing one loopful of the sample from the MIC dilution tubes onto Sabouraud's Dextrose Agar plates an attempt was made to study the fungicidal/ fungistatic nature of the oils. It was observed that all the oils showed the fungicidal (Candidacidal) nature, as no growth was observed on the Sabouraud's Dextrose Agar plates after the incubation period.

**Minimum Killing Time**

An attempt was made to study the time required to kill the pathogens at a particular temperature at MIC level of oils. A variation was observed in Minimum Killing Time of the oils when tested at three different temperatures (**Table – 2**). Immediate killing of *Piper betel* oil was observed against *Candida albicans* at all the three temperatures studied. However, similar effect of *Piper betel* oil was also observed against *Candida glabrata* at $37^0$C and *Candida albicans* at $5^0$C, respectively.

**Effect of temperature and pressure on anti-candida activity of the oils**

This experiment was designed to study the effect of temperature and pressure on the anti-Candida activity of the oils. Oils were heated in a boiling water bath (45, 60, $100^0$C for 1 hour and autoclaved at $121^0$C and 15 lb pressure for 20 minutes) and studied for their anti-Candida activities by Disc Diffusion Method loading MIC values of the oils onto the discs. Surprisingly, a significant increase in the activity of the oils was reported due to heating in case of *Piper betel* and *Nigella sativa* that showed complete inhibition of the test pathogens (pathogens showing poor growth at the centre of the Petri dish and no growth around the discs), on SDA plates.

## Discussion

Three different essential oils were tested for their antimicrobial properties against Candida species. During the investigation we observed lower MIC values of test essential oils against the Candida species which is indicative of their high degree of effectiveness against these pathogens. Observance of lower MIC value of essential oils against both bacteria and fungi is being reported in literature. In contrast to the findings observed in this investigation Rath, et al. (1999) reported a higher MIC values 62.5 and 500 µl / ml of turmeric leaf and rhizome essential oils respectively, against *Candida albicans*. While studying the microbicidal/ microbiostatic nature of these oils, the oils were reported to be microbicidal (Candida-cidal). Microbicidal nature of different essential oils is well documented in literature [12-20].

**Table – 2:** Minimum Killing Time of the Oils.

| **Pathogens** | **Minimum Killing Time of the Oils in minutes** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ***Piper betel*** | | | ***Nigella sativa*** | | | ***Murraya koenigii*** | | |
| | **$5^0$C** | **RT** | **$37^0$C** | **$5^0$C** | **RT** | **$37^0$C** | **$5^0$C** | **RT** | **$37^0$C** |
| *Candida albicans* | 0 | 0 | 0 | 130 | 100 | 110 | 160 | 30 | 10 |
| *Candida glabrata* | 30 | 110 | 0 | 110 | 110 | 100 | 170 | 160 | 20 |
| *Candida tropicalis* | 320 | 160 | 110 | 280 | 50 | 50 | 110 | 40 | 20 |

During the determination of Minimum Killing Time of the oils it was observed that *Piper betel* killed *Candida albicans* immediately at all three test temperatures. Similar results were also observed for *Piper betel* against *Candida glabrata* at $37^0$C. Killing of the Candida species immediately by these oils indicates that the oils cause an irreversible damage to the structure of the test organisms when they come in contact with the oil mixture. Immediate killing of pathogens and irreversible damage to cellular structure by essential oils is well recorded in literature. Since the oils showed activity against Candida spp. at both lower temperatures $5^0$C and $37^0$C, it implies that the activities of these oils are energy independent [21-24].

Energy-independent bactericidal activities of lemongrass, palmarosa and eucalyptus oil against *Escherichia coli* and other bacteria is also recorded. Rath, et al., (2001) reported similar observations while studying the antifungal activity of fractionally distilled and neat turmeric leaf oil. They reported that neat oil killed *Candida albicans*, *Cryptococcus neoformans*, *Trychophyton rubrum* and *Microsporum gypsum* within 1 minute of treatment whereas, 1 hour and 2 hour fractionally distilled oil took a longer time to kill the same pathogens in comparison to neat oil. Our observations here can be agreed with Rath et al., (1999) reported immediate killing of *Candida albicans* and *Cryptococcus neoformans* when treated for a longer time (15 min) in comparison to turmeric leaf oil .

Even on heating the oils at 45, 60, $100^0$C for 1 hour and autoclaved at $121^0$C and 15 lb pressure for 20 minutes, anti-Candidal activity was not lost. Similar properties of various essential oils have been reported by researchers while studying their antimicrobial activities including Candida species. On heat treatment, a significant increase in anti-Candida activity of *Nigella sativa* and *Piper betel* oils was recorded during the investigation. This could be attributable to the change in charge of the compounds present in these essential oils and increase in their mobility.

Further, it suggests that the anti-Candida components that are present in these oils are heat stable and withstand a temperature of $121^0$C and 15 lb pressure, indicating their thermostable and barostable nature. Gupta et al., (2004) reported the persistence of antimicrobial activity of carrot (*Daucus carota*) and celery (*Apium graveolens*) seed essential oils against both Gram positive and Gram negative pathogens after heat treatment ($100^0$C) and autoclaving, indicating the presence of heat stable components in these essential oils as reported in our investigation. Similar results are also reported in literature while studying the antibacterial activity of lime (*Citrus limonum*) and juniper (*Juniperus communis*) oils against 32 strains of methicillin resistant *Staphylococcus aureus*. Das et al., (2009) reported the antibacterial activity of essential oils of three Ocium spp. and their cocktail mixture at high temperature and pressure concluding the presence of heat stable and baro-stable components in essential oils, as reported here in our studies.

Pathogen susceptibility to the oils that are tested may be due to inhibition of cell membrane synthesis, specifically by extracting the sterols from the membrane or inhibiting steroid

synthesis. Senhaji et al., (2007) observed the antibacterial activity of essential oil from *Cinnamum zeylanicum* against *Escherichia coli* 0157:H7 is through outer membrane disintegration and increasing the permeability of ATP through cytoplasmic membrane. Similarly, Rath et al., (2005) also reported the anti-Staphylococcal activity of juniper and lime essential oils against methicillin – resistant *Staphylococcus aureus* (MRSA) through inhibition of cell membrane synthesis. Further, it is to add that the essential oils are rich in terpenes. However, the mode of action of terpenic constituents (essential oils) on microorganisms is not fully understood. But, in view of their hydrophobicity, it is considered that they are involved in mechanism such as permeability of cytoplasmic membrane, coagulation of cell contents and disruption of the proton motive force. Therefore, the anti-Candida activity of these essential oils through membrane inhibition could be attributable to the hydrophobicity of essential oils that enables them to make partitions in the membrane, rendering permeability due to extraction of steroid molecules present on the membrane and leading to leakage of cell contents resulting in death of the cells [25-28].

## Conclusion

With this study we came to a conclusion that anti-Candidal activity of these essential oils against human pathogens is suggestive of their use in pharmaceuticals and cosmetic industries for production of drugs and aroma products. However, further scientific research is essential to investigate the side effects of these oils before consideration of their use.

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